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Regulation of Mitochondrial Processes by Protein S-Nitrosylation

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Abstract

Background—Nitric oxide (NO) exerts powerful physiological effects through guanylate cyclase (GC), a non-mitochondrial enzyme, and through the generation of protein cysteinyl-NO (SNO) adducts— a post-translational modification relevant to mitochondrial biology. A small number of SNO proteins, generated by various mechanisms, are characteristically found in mammalian mitochondria and influence the regulation of oxidative phosphorylation and other aspects of mitochondrial function.

Scope of Review—The principles by which mitochondrial SNO proteins are formed and their actions, independently or collectively with NO binding to heme, iron-sulfur centers, or to glutathione (GSH) are reviewed on a molecular background of SNO-based signal transduction.

Major Conclusions—Mitochondrial SNO-proteins have been demonstrated to inhibit Complex I of the electron transport chain, to modulate mitochondrial reactive oxygen species (ROS) production, influence calcium-dependent opening of the mitochondrial permeability transition pore (MPTP), promote selective importation of mitochondrial protein, and stimulate mitochondrial fission. The ease of reversibility and the affirmation of regulated S-nitros(yl)ating and denitros(yl)ating enzymatic reactions supports hypotheses that SNO regulates the mitochondrion through redox mechanisms. SNO modification of mitochondrial proteins, whether homeostatic or adaptive (physiological), or pathogenic, is an area of active investigation.

General Significance—Mitochondrial SNO proteins are associated with mainly protective, bur soem pathological effects; the former mainly in inflammatory and ischemia/reperfusion syndromes and the latter in neurodegenerative diseases. Experimentally, mitochondrial SNO delivery is also emerging as a potential new area of therapeutics.

Keywords

Metabolism; mitochondria; nitric oxide; respiration; S-nitrosylation; S-nitrosation

Introduction

The mitochondrial nitric oxide (NO) field is still in an early stage even though some 2,000 papers have been published on the subject since the year 2000. The area has generated strong interest mainly for two reasons: first, many fundamental roles for mitochondria have

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been discovered that extend well beyond the capacity for oxidative phosphorylation [1], and second, a rich NO biochemistry that encompasses modifications of peptides and proteins has been found to be important in mitochondrial structure and function [2]. The latter includes interactions of NO with heme (metal nitrosyl; M-NO) and iron-sulfur centers (Fe-S), nitration of proteins ($R\text{-}NO_2$), and the formation of low molecular weight and protein S-nitrosothiols (RSNO), each posing its own set of investigative challenges [3].

These distinct chemistries, stemming from the prototypical activation of guanylate cyclase (GC) [4], a non-mitochondrial enzyme, have important effects on mammalian mitochondrial physiology, pathology, and cell survival [5]. In this paper, the emphasis is on cysteinyl-NO (SNO) proteins [6, 7], which may exist alone or together with NO binding to heme or Fe-S. An appreciation of SNO chemical biology, singly and collectively in the context of the cysteine proteome [8] benefits contemporary investigations of mitochondrial function.

The information on how NO reacts with and regulates various mitochondrial proteins is incomplete, and as discussed later, has generated some controversy about the roles these fascinating organelles play in cellular energy and redox regulation, heme biosynthesis, calcium and iron homeostasis, and apoptosis [9–11]. Moreover, NO comes into play in the process of mitochondrial quality control, which involves more than 1,000 proteins and encompasses biogenesis, proliferation, and autophagy (mitophagy) through integration of nuclear and mitochondrial gene regulatory and assembly mechanisms [11]. These processes regulate the cell's functional capacity (e.g. mitochondrial volume and phenotype) and impinge on key aspects of cellular stress resistance and senescence for which mitochondria are increasingly recognized to have a role [12].

The ability to trap and measure SNO-adducts in cells and organelles is challenging because SNO proteins are labile, and reversibility of NO ligation is fundamental to SNO signal transduction [13–17]. This has been a problem since the former controversy over SNO-hemoglobin [18]; nonetheless SNO is associated with the functions of more than 100 proteins [19]. The analytical methodology is covered elsewhere in this issue, and here attention is given mainly to its limitations, particularly in the context of active denitrosylating systems involving S-nitrosoglutathione reductase (GSNOR/ADH Class3) and thioredoxin-2 (Trx2). In order to emphasize the signal transduction aspects of this post-translational modification, I have also used the —S-nitrosylation nomenclature (in lieu of S-nitrosation) to describe the covalent addition of NO to RSH (more correctly to thiolate anion, RS^-).

Finally, it is well appreciated that SNO signaling overlaps with the non-specific overproduction of both SNO and other reactive nitrogen species (RNS) during nitrosative stress [20]. This too has been a significant issue experimentally because it requires the nuanced definition of conditions and meticulous and often laborious in situ evaluation of mitochondrial function, and therefore remains much in need of further research. In order to be concise, I have chosen here to focus on sources of mitochondrial NO, the mitochondrial SNO targets, their relationship to mitochondrial glutathione (GSH), and mitochondrial SNO protein functionality in health and disease.

Sources of NO in Mitochondria

Three difficult, but often asked questions on NO in mitochondria have been the center of attention: 1) where does the NO come from; 2) how much NO, if any, do mitochondrial proteins contain physiologically; and 3) when does mitochondrial NO become toxic? The answers generally depend on four factors: the type, location, and function(s) of the protein, and the specific modifications to which it is subject. NO modifications are part of the larger cysteine proteome [8, 21, 22] in which cysteine residues, in addition to disulfide and SNO

formation, may undergo other modifications. The addition of GSH to cysteine (glutathionylation or glutathiolation) — often following SNO formation — is a protective, reversible modification. Other oxidative modifications include the formation of sulfenic acid, which can be further oxidized to sulfinic acid (S^{+2}) and then to biologically irreversible sulfonic acid (S^{+4}). Protein cysteines also interact with fatty acids, most often palmitate — called S-acylation or palmitoylation, the catalytic formation of fatty acid esters that produce various functional effects [22]. Finally, hydrogen sulfide (H_2S) generated endogenously from free L-cysteine can attach another sulfur to cysteine, yielding the protein hydropersulfide (−SSH). Sulfhydration often involves the same cysteines as S-nitrosylation and often activates the protein.

Mitochondrial NO Generation

The issue of where mitochondrial NO comes from has perhaps generated the most intense interest as well as the most controversy in the field. There is little argument that mitochondria contain bioactive NO— they do, as determined by the presence and formation of nitrosyl heme and copper [23], nitrated proteins [24], and S-nitrosothiols [25] as well as by SNO clearance or denitrosylating mechanisms [26].

The first and most widely appreciated effect of NO on mitochondria is its ability to inhibit cytochrome *c* oxidase (Complex IV), the terminal acceptor of the electron transport chain (ETC) that reduces O_2 to water [27–29]. It is thus important to know whether NO is actually generated by mitochondria or diffuses into them, and whether it regulates or simply inhibits respiration. Many investigators have reported that mitochondria synthesize NO from L-arginine [30–39], e.g. by a mitochondrial NO synthase (mtNOS); however, others have maintained that this is a broken cell artifact [40–44]. The issue is important for two reasons: first is enzyme regulation and second is the sizeable superoxide ($^{\cdot}O_2^-$) leak from the electron transport chain [45]— as the chemical combination of NO and $^{\cdot}O_2^-$ produces highly toxic peroxynitrite anion ($ONOO^-$), which decomposes to NO_2 and an OH radical species [46, 47].

For more than 15 years, there have been reports that one of the isoforms of NO synthase (NOS) is resident in mitochondria. NOS was first reported in liver and rat brain mitochondria in 1995 by Bates *et al.* who detected neuronal NOS (nNOS; NOS1) by immunochemistry [48]. Subsequent groups have reported NO production, calcium-dependent NOS activity, and NOS localization to the inner mitochondrial membrane— often in liver mitochondria [33, 36, 39, 43, 49–52]. Calcium-induced NO release was also reported in cardiac mitochondria of wild-type mice that was absent in $nNOS^{-/-}$ mice [33].

Subsequent efforts to identify a specific gene for mtNOS failed, but NO production in a number of cases has been attributed to nNOS (NOS1) found in association with mitochondria under some, but not all conditions [39]. The nNOS transcript is an α splice variant [51], and the protein is constitutive to the inner membrane [51], despite having no known mitochondrial leader sequence or other importation mechanism. In liver and brain mitochondria of rats, it has been observed that mtNOS is not detectable by nNOS antibodies to the PDZ domain at the N-terminus, but is detectable by antibodies to C-terminal domains [52, 53]. On this basis, cleavage of this domain has been proposed to enable mitochondrial importation and to account for a lower molecular weight of the protein.

Of further interest is that functional nNOS is often detected in association with heart mitochondria [54] and conditional nNOS over-expression in the mouse heart is connected with Hsp90-associated accumulation of mtNOS, decreased ROS production, and a smaller infarct size after ischemia/reperfusion (IR) [55]. Both iNOS (NOS2) and eNOS (NOS3)

have also been detected in mitochondria under normal and under pathological conditions [56–60], such as acute inflammation [61, 62].

Functionally, mtNOS has been proposed to regulate mitochondrial O_2 utilization by the reversible formation of the NO-Cu^+_B moiety of cytochrome *c* oxidase [49, 52], which derives from the observations that nanomolar NO reversibly inhibits the oxidase [2, 29]. Mitochondrial NOS would thus slow O_2 consumption and oxidative phosphorylation, for example, through a NOS substrate, co-factor, or calcium-regulated mechanism. However, this hypothesis has not been tested under strictly physiological conditions.

The activity of mtNOS would require a higher O_2 concentration in mitochondria than generally occurs [63], assuming a Km for O_2 comparable to nNOS, which is two orders of magnitude higher than that of cytochrome oxidase [64]. Also, mitochondrial superoxide production increases at both high [65] and low [66] PO_2, and the generation of reactive nitrogen species, such as $ONOO^-$, indiscriminately inactivates proteins, Fe-S clusters, nucleic acids, and lipids [67]. $ONOO^-$ reacts rapidly with thiols to produce thiyl radicals or sulfenic acids, which usually react with GSH or other −SH groups to form disulfides [68]. The effects of $ONOO^-$ are generally rapid, non-specific, inhibitory, and irreversible [69]. This NO toxicity along with technical concerns about mitochondrial purity, low-specificity NOS activity measurements, and non-optimized controls have helped sustain the mtNOS controversy [42–44].

Given that a number of laboratories have observed mtNOS, one must seek to escape this apparent conceptual corner. There are recognized strategies of targeted NOS translocation or tightly restricted NOS regulation, but mitochondria may also dispose of NO harmlessly, for instance, by exporting it as low molecular weight SNO or by converting it to a metabolite such as nitrite (NO_2^-). Cytochrome oxidase will consume NO in this way when its redox centers are oxidized and electron flow is available [70]. The inactivation of NO in cells depends on the NO: O_2 ratio, and it has been suggested that at low O_2 concentrations, when cytochrome oxidase is reduced, NO is diverted towards GC to help activate hypoxic vasodilation [71], thereby improving O_2 availability at the mitochondria.

External Sources of Mitochondrial NO

Mitochondrial NOS aside, NO can gain entrance to mitochondrial compartments in other ways. The fact that NO lacks an electronic charge and is soluble in hydrophobic environments allows it to diffuse across biological membranes and signal in a paracrine manner at a short distance from its site of generation [72]. Moreover, in cells and tissues, NO generated by calcium-regulated nNOS or eNOS, or by iNOS induction, or by NO donors significantly affects mitochondrial function, but the extent to which this is physiological, pathological, or pharmacological is open to discussion [73].

Bioactive NO can also enter mitochondria as low molecular weight SNO compounds, such as cysteinyl-NO (CSNO) or nitrosoglutathione (GSNO), or as nitrosylated proteins. Peptide cysteine residues participate in Cys-to-Cys NO transfer or transnitrosation reactions that may involve cytoplasmic-to-mitochondrial communication. Although NO_2^- can be produced from NO by cytochrome oxidase, the reverse reaction, NO production from NO_2^- by heme-based nitrite reductase activity [74] might generate NO in hypoxic mitochondria without interfering with O_2 utilization. This speculation, however, has not been tested experimentally in mitochondria at physiological nitrite concentrations.

Under some conditions, iNOS associates with mitochondria either because it is produced in the cytosol in high abundance or because it is targeted to the outer mitochondrial membrane for a specific purpose [59, 60, 62]. During sepsis in mice, for instance, much of the iNOS in

hepatocytes associates with the outer mitochondrial membrane [62]. Endothelial NOS may also attach to the outer mitochondrial membrane during periods of enzyme induction [56].

Targets of Mitochondrial Protein S-nitrosylation

The effort that has gone into the identification of mitochondrial targets of S-nitrosylation has had variable results, and evidence for SNO functionality derives largely from tissues with high ATP utilization rates, such as the heart, brain, and liver. Attempts to identify the SNO-protein population in intact mitochondria indicate that SNO is present in a small subset of proteins and that roughly 1% of the mitochondrial proteome is available for S-nitrosylation [75]. This suggests that mitochondrial SNO proteins formed under cell stress or by injury, or in response to low levels of exogenous S-nitrosylating agents, are candidates for physiological regulation. It is assumed that identifying and characterizing these targets will inform how S-nitrosylation is involved in mitochondrial homeostasis and when SNO aberrancy— under- or over-production— causes pathology. Moreover, mitochondrial-targeting S-nitrosylating agents have been protective in several disease models [75–78].

It is no surprise that the complement of mitochondrial SNO-proteins detected in NO donor experiments varies with biological preparation, donor type and concentration, tissue PO_2, and other factors [79]. The analytical procedures used in most studies, *e.g.* biotin switch or labeling with fluorescent probes, may not detect low abundance proteins and do not allow specific cysteine residues to be identified, which require more sensitive and specific approaches, such as differential alkylation, to precisely locate the modified cysteines [8]. The most sensitive proteomic screening method available to date involves SNO-DIGE (difference in gel electrophoresis), which detect low concentrations of SNO proteins on 2D gels [80]. A resin-assisted method has also been developed to capture SNO proteins and compare them against simultaneous oxidation of cysteines in the same proteins [81] A list of mitochondrial proteins that undergo SNO formation is provided in Table 1, a few of which are discussed briefly below.

Complex I and Other ETC Components

Mitochondria utilize four ETC complexes (I-IV) and the ATP synthase (Complex V) to carry out oxidative phosphorylation. Studies of the effects of NO on the ETC actually originated nearly 50 years ago with cytochrome *c* oxidase [82]. Historically, because its chemical behavior resembles that of O_2, NO has been used as a probe, like CO, to inhibit O_2 utilization by the oxidase [28]. Brief exposures to low concentrations of NO promptly and reversibly inhibit cytochrome oxidase [83]. NO interacts with both the oxidized and reduced oxidase complex, and depending on the redox states of the four centers, are of two main types; one is competitive with O_2 (like CO), while the other is non-competitive [84].

At a physiological PO_2, ~60 nM NO is required to inhibit cellular respiration by ~50% [85]. Thus, at a normal PO_2, cytochrome oxidase is 50-fold less sensitive to NO than is GC. In order for NO generated physiologically in the cytoplasm to inhibit cytochrome oxidase, GC presumably would be almost fully activated. Hence, whether physiological NO actually regulates respiration at cytochrome oxidase when vascular GC is not already activated is an unresolved question [2, 86, 87]. Nevertheless, a variety of NOS-expressing cells do show NO-mediated suppression of respiration by some mechanism [2, 86–89].

NO has also long been known to inhibit Complex I [90]. In cells deficient in mitochondrial superoxide production, NO inhibits both Complexes I and IV [91, 92]. Unlike Complex IV inhibition, which may remain reversible, Complex I inhibition easily becomes irreversible due to oxidation and nitration of susceptible subunits of the complex [93]. Complex I inhibition by NO also increases as reduced glutathione (GSH) concentration decreases in the

cell [91, 94], which provided early evidence of roles for S-nitrosylation and for glutathionylation [91, 93–96].

Mammalian Complex I is composed of 45 different subunits [97], which include a single flavin mononucleotide and eight iron-sulfur groups [98] that catalyze a two-electron transfer from NADH to ubiquinone with the movement of $4H^+$ across the inner mitochondrial membrane [99]. Complex I is inactivated by SNO in several ways, but in most cases, inactivation can be reversed with dithiothreitol, copper/ascorbate, or by exposure to light, all consistent with a role for S-nitrosylation [93]. Moreover, many SNO compounds reversibly inactivate Complex I even if little or no free NO is available, implying the involvement of transnitrosation [91, 94].

A property of Complex I called the active/deactive (A/D) transition causes a delay in the onset of NADH:ubiquinone oxidoreductase activity or its reverse [100]. Deactive or dormant (D) enzyme is reactivated by stimulation of enzyme turnover [100]. The active (A) form is resistant while the D-form is sensitive to inactivation by SNO [93], but this may be relevant only under pathological conditions, when enzyme turnover is limited by hypoxia or by a high NO: O_2 ratio [93, 101]. A critical thiol has been identified in the catalytic region of Complex I by fluorescence labeling and proteomic analysis at Cys-39 of the mitochondrial-encoded ND3 subunit in bovine heart mitochondria [102]. Cys-39 is found in a loop connecting the first and second transmembrane helix of ND3 that may be a specific site of physiological SNO regulation.

Metabolic Pathways

Apart from Complex I, other metabolic enzymes can be inhibited by S-nitrosylation, such as mitochondrial aldehyde dehydrogenase (ALDH2) [103] and aconitase [104]. Using a mitochondria-targeted S-nitrosothiol (MitoSNO) and SNO-DIGE, one study reported S-nitrosylation of eight enzymes of carbohydrate and fatty acid metabolism in rat heart mitochondria [80]. Five are involved in fatty acid β-oxidation including the very-long-chain acyl-CoA dehydrogenase, the short-chain acyl- CoA dehydrogenase, and enoyl-CoA hydratase. Also identified were carnitine palmitoyl transferase 2, which initiates β-oxidation, and the electron-transferring flavoprotein dehydrogenase, which transfers electrons derived from the oxidation of fatty acids to ubiquinone. Three citric acid cycle enzymes were also S-nitrosylated: aconitase, α-ketoglutarate dehydrogenase, and the succinate dehydrogenase flavoprotein (subunit A), a part of Complex II. The activity of three of these eight enzymes — aconitase, α-KGDH and ALDH2— was checked and found to be inhibited by SNO.

The investigators also postulated that decreased Complex I activity by S-nitrosylation may limit cardiac mitochondrial ROS production and calcium overload during ischemia-reperfusion (IR) [94, 105, 106]. They noted that temporary inhibition of the citric acid cycle and β-oxidation may prevent NADH and reduced ubiquinone from building up and creating a burst of ROS on reperfusion that would damage mitochondria [107]. It is also thought that S-nitrosylation may directly prevent irreversible protein inactivation by ROS [78, 81] or by promoting glutathionylation [108–112].

The effects of NO on the cytosolic and mitochondrial isoforms of the branched-chain amino-acid aminotransferases (BCATc and BCATm, respectively), which control the first step in the catabolism of essential branched chain amino acids, have also been explored [113]. Several NO donors including GSNO inactivate both human isoforms in a dose-dependent manner. Low GSNO concentrations cause a time-dependent inhibition of BCAT that correlates with loss of thiol groups. Although GSNO inactivates both, S-nitrosylation of the cytosolic, but not the mitochondrial isoform was found, suggesting differential regulation by NO. Reversal of GSNO-BCAT with GSH alone was limited, but full reactivation was

possible with GSH/glutaredoxin, adding support to the physiological denitrosylation hypothesis.

The Mitochondrial Permeability Transition Pore (MPTP)

When mitochondria are overloaded with calcium, the inner mitochondrial membrane becomes permeable to solute and to molecules of <1.5 kDa because of the opening of a non-selective mitochondrial permeability transition pore (MPTP) [114]. Opening of the MPTP is facilitated by elevated phosphate concentrations, depletion of adenine nucleotides, and/or oxidation of critical thiols associated with the pore [115]. Pore opening uncouples oxidative phosphorylation and worsens ATP depletion, calcium homeostasis, and oxidative stress, leading to cell death by necrosis, apoptosis, or by intersecting mechanisms [116]. Pathologically, energy failure, ROS production, and calcium overload are major interactive factors in MPTP opening in IR injury of the heart [115], brain [117], and liver [118], while in the heart, pre-conditioning dampens these triggers and reduces the sensitivity of the MPTP to calcium [119].

Although the behavior of the MPTP is fairly well elucidated, its molecular composition is not. There is a role for cyclophilin-D (CyP-D)— a peptidyl-prolyl cis-trans isomerase involved in calcium regulation— for which hyper-activation most likely provokes a conformational change in inner membrane proteins at the pore site [120]. Also involved in pore regulation, but not a direct constituent, is the adenine nucleotide translocase (ANT) [121], which allows ADP and ATP to inhibit MPTP opening [122]. The mitochondrial phosphate carrier (PiC) has also been implicated in pore formation, and Halestrap has suggested that calcium triggers a conformational change in the PiC that is facilitated by CyP-D [123]. Agents that modulate the MPTP interact with critical thiol moieties on either or both the PiC and the ANT.

The effects of NO on the MPTP are variable, and it is unclear whether SNO plays a role in physiological MPTP regulation [124]. In general, low NO concentrations delay pore opening in response to calcium [38, 125], while higher concentrations behave like strong oxidants that facilitate pore opening [35, 126]. In studies to evaluate how pharmacological manipulation of the MPTP affects ischemic damage, NO was protective, but this involved both direct and indirect mechanisms [127–129]. In the heart, over-expression of iNOS inhibits MPTP opening during IR [130]. In other cases, SNO enhances MPTP opening— most notably in models of disease where the specificity of interventions for the MPTP is low due to SNO effects on other aspects of mitochondrial function [131, 132]. Moreover, pharmacological and perhaps physiological SNO levels have important non-mitochondrial effects that may impact MPTP function, for instance, through altered Ca^{++} regulation by the sarcoplasmic/endoplasmic reticulum Ca-ATPase (SERCA) [133–135].

Chaperonins

Most mitochondrial proteins are synthesized as cytoplasmic pre-proteins and imported across the outer and inner membrane by translocases and chaperonins that guide the entry, processing, and folding of the peptides [136]. These import systems are crucial for mitochondrial quality control and for mitochondrial biogenesis [137]. NO activates mitochondrial biogenesis [138], which improves the physiological responses to increased ATP demand and to increased turnover of mitochondria [139]. However, the extent to which NO contributes to mitochondrial protein importation is only now being investigated.

In systemic inflammatory states, such as sepsis, a diminished capacity for oxidative phosphorylation from ETC damage occurs in which NO production by iNOS may contribute [140]. Moreover, increased demand for ATP is met by adjusting energy management and by

increasing the rates of mitochondrial biogenesis and mitophagy. Mitochondrial biogenesis involves NO and is not fully met in sepsis in iNOS$^{-/-}$ mice, which display impaired hepatic recovery of mtDNA copy number and peak respiration rates [62, 141]. The absence of iNOS in sepsis is associated with failure to increase the levels of mitochondrial transcription factor-A (Tfam) and DNA polymerase gamma (Polγ) during mitochondrial biogenesis [62]. The iNOS binds to the outer mitochondrial membrane and S-nitrosylates heat shock proteins (Hsp) 60 and Hsp70 (mortalin), which are involved in the importation and folding of mitochondrial proteins translated in the cytoplasm [142]. The iNOS specifically regulates Tfam binding to Hsp 60 through S-nitrosylation of Cys-237, which leads to increases in mitochondrial levels of the transcription factor.

Mitochondrial Fission Proteins

Mitochondria undergo synchronized structural modifications— known as fission and fusion — that regulate their numbers and size, and the supply of energy to different parts of the cell [143]. Recent discoveries have indicated that excessive fission can be triggered in neurons by NO-related dysfunction of the fission-inducing dynamin-related protein-1 (Drp1) on the outer mitochondrial membrane [144]. Drp1 contains an N-terminal GTPase domain and a C-terminal GTPase effector domain (GED) involved in homodimer formation and GTPase activation that is necessary for fission [145].

In neurons, NO induces mitochondrial fission, and S-nitrosylation of Drp1 activates both the GTPase and fission. Drp1 contains nine cysteines, each of which has been mutated and the constructs transfected into cells [11, 144, 145]. Of the nine, only Cys-644 mutation in the GED domain decreased SNO-Drp1 formation. In neurons, nNOS activation leads to SNO-Drp1 formation, which is absent in Drp1-Cys-644 mutant cells, but present in the other mutants. Drp-1-Cys-644 mutant over-expression prevented NO-mediated mitochondrial fragmentation while the other mutations did not, indicating that Cys-644 is the critical Drp1 S-nitrosylation site. Further evidence supports that mitochondrial fragmentation contributes to synaptic damage and neuronal death through increased nitrosative and oxidative stress and bioenergetic compromise. This fragmentation process, along with other NO-dependent excitotoxic events, has been associated with neurodegenerative conditions, such as Alzheimer's disease [146].

Role of the Mitochondrial Glutathione System

NO and Glutathionylation

As mentioned earlier, NO promotes glutathionylation— the reversible post-translational addition of GSH to protein thiols [147–149]. Formation of these mixed disulfides is involved in redox regulation of protein structure and function. Mitochondria contain millimolar GSH, which is acquired largely through organic anion exchangers [150]. The reduced GSH pool in mitochondria is typically high because GSH oxidation to the disulfide (GSSG), for instance, by mitochondrial glutathione peroxidase, leads to prompt reduction by mitochondrial glutathione reductase or to the export of GSSG to the cytosol [151].

Many mitochondrial proteins contain critical thiol residues, and mitochondria contain detectable S-nitrosoglutathione (GSNO), which forms by direct interaction of NO with GSH or by transnitrosation reactions with other S-nitrosylated proteins [152]. Studies of proteins containing reactive sulfhydryls indicate that micromolar NO or GSNO will reversibly generate S-nitrosylated and/or S-glutathionylated forms, lending support to redox-regulation or redox-switch hypotheses [91, 111, 148, 149, 153]. Similar reversibility can be obtained in cells by exposing them to GSNO or other NO donors at low concentrations. This has been demonstrated, for example, for the cytoplasmic glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [148]. Thiol residues on GAPDH were S-nitrosylated

by the NO donor nitrosonium tetrafluoroborate (BF4NO), which inhibited the enzyme activity. This SNO decomposed spontaneously, restoring activity. In cells, GSNO promoted glutathionylation of GAPDH and enzyme inhibition was reversed by reduction with dithiothreitol [148]. Accordingly, glutathione disulfide (GSSG) also reversibly inactivates certain enzymes by S-glutathionylation, e.g. creatine kinase [110]. It has also been suggested that cytochrome *c* may catalyze GSNO formation well enough to inhibit Complex I [154].

The data on ETC damage by NO and its mitigation by GSH [91, 155] has given rise to the view that S-glutathionylation safeguards mitochondrial proteins from oxidation/nitration and regulates cell metabolism. Glutathionylation blocks cysteine oxidation by preventing the formation of disulfides and higher oxides of sulfur that may be irreparable. In the presence of NO, the GSH content also strongly influences the amount of $ONOO^-$ and other oxidants that is detected; for example, GSH depletion renders rat hepatocytes more sensitive to NO toxicity [156]. Protein thiols in mitochondrial membranes are more easily S-nitrosylated by low molecular weight SNO than by NO or $ONOO^-$, and they are reduced by GSH— tending to endure mainly when the mitochondrial GSH pool is oxidized or depleted [94].

The importance of GSH protection is illustrated by the sensitivity of citric acid cycle aconitase to inactivation by $ONOO^-$. Low micromolar $ONOO^-$ inactivates aconitase by reducing the Fe-S cluster to an inactive form with depletion of labile iron. The $ONOO^-$ concentration for half-inhibition is greatly increased by citrate binding to the active site, and the enzyme is inactivated by the nitration of Tyr-151 and -472 and the oxidation of Cys-126 and -385 to sulfonic acid [69]. The latter cysteine binds the Fe-S cluster and the other residues are nearby, suggesting these modifications disrupt the active site. Aconitase is also reversibly inhibited by glutathionylation, suggesting this as a mode of modulating citric acid cycle activity and/or preventing a permanent failure [69, 104].

There are also differences in the NO-glutathione effect on mitochondrial function in different cell types. For instance, astrocytes display lower sensitivity to NO toxicity than neurons in part because of up-regulation of GSH synthesis [157]. Exposure of primary cortical neurons or astrocytes to a steady flux of NO (~0.25 microM/sec) decreases GSH and increases GSNO and GSSG formation. Dose-dependent oxidations and correlations between cell viability and redox state are observed. Neurons also show a higher sensitivity to NO than do astrocytes due to a lower capacity to recover GSH through glutathione reductase [158].

Glutathione and denitrosylation

Mitochondria also perform enzymatic denitrosylation [26] and deglutathionylation [108, 159], indicating tight regulation of these processes. The mitochondrial GSH-dependent oxidoreductase glutaredoxin-2 (Grx-2) helps maintain redox homeostasis through the efficient catalysis of both mono- and di-thiol reactions [160]. Grx-2 has a high affinity for protein-glutathione disulfides, but also accepts electrons from thioredoxin reductase, allowing it to support mono- and di-thiol detoxification. Compared with cytosolic Grx-1, mitochondrial Grx-2 retains activity after oxidant exposure, does not form inactive disulfide homodimers, and is relatively resistant to S-nitrosylation [161].

The involvement of GSH in protein denitrosylation enables the attainment of a complete cycle of S-nitrosylation and denitrosylation as shown in Figure 1. Several enzymes can reduce SNO and are thus physiological denitrosylases (see [162]). The prototype is the cytosolic GSNO reductase (GSNOR/ADH Class 3), which uses NADH to convert GSNO to GSNHOH and then to GSSG [163]. The cycle is completed by reduction of GSSG to GSH by glutathione reductase [164]. GSNOR acts on GSNO, but not on SNO proteins, yet affects

the equilibrium between GSNO and the SNO-protein pools and helps set SNO protein levels in the cell.

By comparison, the thioredoxins (Trx1 and Trx2) lack GSNOR's stringent substrate specificity and thus mediate denitrosylation of many SNO proteins [26]. Following denitrosylation, reduced Trx is regenerated by Trx reductase (TrxR) at the cost of NADPH. Reduced Trx also provides electrons to methionine sulfoxide reductase for repair of methionine sulfoxide residues and to peroxiredoxins for detoxification of H_2O_2 and $ONOO^-$ [164]. A key concept is that the level of protein S-nitrosylation is not determined simply by the rate of SNO generation, but by a local balance among S-nitrosylation, denitrosylation, and -SH protection. Accordingly, protein denitrosylation is an active part of SNO-based signal transduction.

Functionality of Mitochondrial S-nitrosothiols: Physiological Regulation vs. Toxicity

The previous sections summarized the main evidence that mitochondrial S-nitrosothiols play important physiological and adaptive roles in cell stress and that SNO deregulation is involved in the pathogenesis of certain diseases. It should be reiterated that a conceptual difficulty has been a lack of a clear demarcation between primary physiological mechanisms of regulation and secondary responses to NO toxicity for some mitochondrial functions. Several examples have been cited, such as inhibition of Complex I in cardiac IR, S-nitrosylation of Hsp60 in sepsis, and deregulation of mitochondrial fission in neurodegenerative disease. In each case, problems may arise due to multiple inputs to the SNO —on and off mechanisms and the possibility that SNO regulation fails— on or off— in disease.

In the heart, where S-nitrosylation is closely connected to protection from IR injury, it is known that certain pharmacological agents, such as the nitro-vasodilator nitroglycerin, are activated in mitochondria and lead to SNO protein accumulation [162]. This implies a protective role for S-nitrosylation, as seen during preconditioning [165]. The delivery of S-nitrosylating agents to mitochondria is also protective in cardiac IR possibly through the inhibition of ROS generation at Complex I [75]. Other examples include S-nitrosylation of cardiac proteins involved in protective estrogen receptor activation [166] and statin-mediated S-nitrosylation and activation of thioredoxin, resulting in less cellular damage by ROS [167].

The heart can be protected from ischemia by a brief period of ischemic preconditioning (IPC), which increases NO generation [168]. The data suggest that IPC results in protein *S*-nitrosylation, which contributes to cardiac protection. An increase in mitochondrial SNO protein content in pre-conditioned rat hearts has been postulated to improve cell survival by regulating respiration, redox state, and/or the MPTP [105, 169]. The induction of iNOS may also contribute to the protective effects of IPC through mitochondrial SNO generation [130, 170].

Multiple mitochondrial enzymes that are inhibited by S-nitrosylation, such as Complex I and citric acid cycle enzymes, show increased S-nitrosylation after IPC, while others require an additional S-nitrosylating agent. Exactly how such combinations of SNO proteins protect against cardiomyocyte cell death remains under investigation, but a temporary interruption of respiration may prevent excessive generation of superoxide and $ONOO^-$ in early reperfusion and alleviate harmful thiol oxidation that might permanently inactivate metabolic enzymes [81] or permit MPTP opening. A SNO-block at Complex I would prevent reducing equivalents as NADH generated by the citric acid cycle from entering the

ETC while oxidation of $FADH_2$ from succinate and fatty acids (the preferred substrate of the heart) could continue. The accumulation of matrix NADH could hypothetically support mitochondrial NADPH generation and mitochondrial antioxidant defenses. If however, S-nitrosylation also blocks pyruvate and fatty acid oxidation [80], oxidative phosphorylation would be interrupted and mitochondrial PO_2 would rise. Such deep interference with cellular ATP provision, if widespread, must be reversed quickly in order to avoid cell death and permanent loss of contractile function.

Conclusions

Evidence for a physiological role for SNO formation in the regulation of a small but significant number of mitochondrial proteins is steadily accumulating, but there is still some uncertainty about how SNO is generated, reacts with, and regulates these and perhaps other proteins. Persuasive evidence for SNO regulation of mitochondrial functions comes from studies on inhibition of Complex I and steps in intermediary and fatty acid metabolism, the activation of mitochondrial protein importation, and in neurons, the regulation of mitochondrial fission. The presence of both S-nitrosylating and denitrosylating mechanisms and the readiness of SNO reversibility lends rationale to redox-regulation hypotheses for mitochondria, especially in tissues with high energy requirements. Although there is not yet a cohesive mitochondrial SNO story, the evidence implies that an adaptive —SNO-block protects the organelle under stress, perhaps as an anti-oxidant strategy or as a prelude to mitochondrial fission and/or biogenesis. A fuller understanding of mitochondrial SNO in the future will predictably improve our insights into cell physiology and pathology as well as reveal new avenues of mitochondrial therapy.

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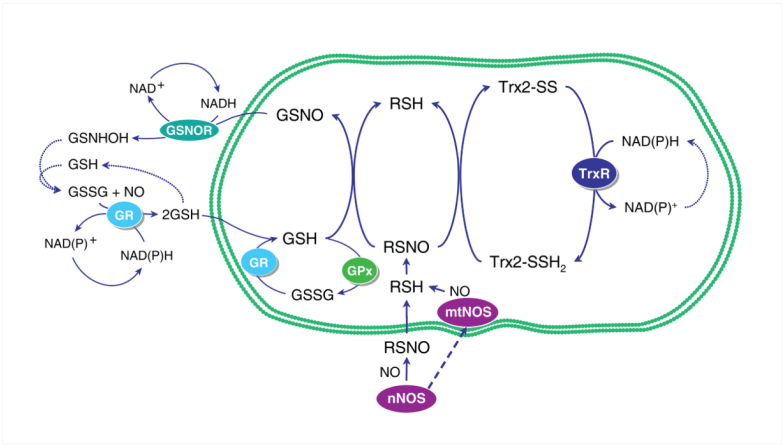

Figure 1.

S-nitrosylation and denitrosylation of mitochondrial proteins with available −SH groups. The transnitrosylation of glutathione (GSH) by S-nitrosylated proteins (RSNO) generates GSNO and the unmodified protein (RSH). The thioredoxin-2 (Trx2) system is shown inside the mitochondrion coupled to protein denitrosylation. Oxidized Trx2 is then reduced by thioredoxin reductase (TrxR2), which consumes NADPH to regenerate active Trx2. The GSNOR cycle is shown in the cytoplasm putatively linked to mitochondrial protein denitrosylation in the context of glutathione recycling (Glutathione peroxidase; GPx and glutathione reductase; GR). GSNO is reduced by GSNOR to generate GSNHOH, which further reacts with GSH to generate GSSG. The cycle is completed by GSSG reduction to GSH by GR.

Table 1
Mitochondrial Proteins Sensitive to In Situ S-nitrosylation

A small number of mitochondrial proteins with critical thiol moieties are known to undergo SNO adduct formation in various species including the mouse, rat, and human. The table lists 25 major SNO proteins and their main functions, accession numbers for the human protein from the Protein Knowledgebase (UniProtKB/Swiss-Prot) website, and reference numbers. Note seven of eight citric acid cycle enzymes and five fatty acid oxidation enzymes have been reported to undergo S-nitrosylation.

Accession Number	Protein	Function	SNO Effect	Reference
Q99798	Aconitase	Citric acid cycle	Inhibitory	75, 77, 78,101
O75390	Citrate synthase (CS)	Citric acid cycle	?	78
Q02218	2-oxoglutarate dehydrogenase	Citric acid cycle	Inhibitory	75, 77
P48735	Isocitrate dehydrogenase	Citric acid cycle	?	77, 78
P40926	Malate dehydrogenase	Citric acid cycle	?	76, 77, 78
Q9P2R7 P53597	Succinyl CoA ligase Subunits α and β	Citric acid cycle	?	78
P09622	Dihydrolipoyl dehydrogenase	PDH complex	?	76
P03897	NADH dehydrogenase subunit 3	ETC Complex I	Inhibitory	99
P31040	Succinate dehydrogenase A (SDHA)	ETC Complex II Citric acid cycle	?	77, 78
P31930	Cytochrome b-c1 complex subunit 1	ETC Complex III	?	77, 78
P49748	Very long chain acyl-CoA dehydrogenase (VLCAD)	Fatty acid oxidation	?	77, 78
P16219	Short chain acyl-CoA dehydrogenase	Fatty acid oxidation	?	77
P23786	Carnitine palmitoyl transferase 2	Fatty acid oxidation	?	77
P30084	Enoyl CoA hydratase	Fatty acid oxidation	?	77, 78
Q16134	Electron transferring flavoprotein dehydrogenase	Ubiquinone oxidoreductase	?	77, 78
P05091	Aldehyde dehydrogenase-2 (ALDH2)	Alcohol metabolism	Inhibitory	100
O15382	Branched chain amino acid aminotransferase (BCAT2)	Amino acid catabolism	Inhibitory	110
P10809	Heat shock protein 60 (Hsp60)	Chaperonin	Activating	59
P38646	Heat shock protein 70 (mortalin)	Chaperonin	Activating	59
O00429	Dynamin-related protein-1 (Drp1)	Mitochondrial Fission	Activating	142
Q9UL12	Sarcosine dehydrogenase	Sarcosine degradation	?	76
P54868	Hydroxymethylglutaryl CoA synthase	Mevalonate biosynthesis	?	76
P00505	Aspartate aminotransferase	Amino acid exchange	?	78
P45880	VDAC2	Voltage dependent anion channel	?	78